

The Unwinding of Duplex Regions in DNA by the Simian Virus 40 Large Tumor Antigen-associated DNA Helicase Activity*

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The DNA helicase activity associated with purified simian virus 40 (SV40) large tumor (T) antigen has been examined. A variety of DNA substrates were used to characterize this ATP-dependent activity. Linear single-stranded M13 DNA containing short duplex regions at both ends was used to show that SV40 T antigen helicase displaced the short, annealed fragment by unwinding in a 3' to 5' direction. Three different partial duplex structures consisting of 71-, 343-, and 851-nucleotide long fragments annealed to M13 single-stranded circular DNA were used to show that SV40 T antigen can readily unwind short and long duplex regions with almost equal facility. ATP and MgCl₂ were required for this reaction. With the exception of GTP, dGTP, and CTP, the other common nucleoside triphosphates substituted for ATP with varied efficiency, while adenosine 5'-O-(thiotriphosphate) was inactive. The T antigen helicase activity was also examined using completely duplex DNA fragments (~300 base pairs) with or without the SV40 origin sequence as substrates. In reactions containing small amounts (0.6 ng) of DNA, the ATP-dependent unwinding of duplex DNA fragments occurred with no dependence on the origin sequence. This reaction was stimulated 5- to 6-fold by the addition of the *Escherichia coli* single-stranded DNA-binding protein. When competitor DNA was added so that the ratio of SV40 T antigen to DNA was reduced 1000-fold, only DNA fragments containing a functional SV40 origin of replication were unwound. This reaction was dependent on ATP, MgCl₂, and a DNA-binding protein, and was stimulated by inorganic phosphate or creatine phosphate. The origin sequence requirements for the unwinding reaction were the same as those for replication (the 64-base pair sequence present at T antigen binding site 2). Thus, under specified conditions, only duplex DNA fragments containing an intact SV40 core origin were unwound. In contrast, unwinding of partially duplex segments of DNA flanked by single-stranded regions can occur with no sequence specificity.

The replication of SV40 DNA depends upon the specific interaction between the SV40 core origin (ori⁺) sequence and the SV40 large T antigen, the only viral protein essential for SV40 DNA replication (1). The development of an *in vitro* system capable of replicating SV40 DNA in an origin-dependent reaction has provided an opportunity to examine the molecular events involved in replication of this viral DNA in detail (2-4). Replication initiates at the viral origin and proceeds bidirectionally both *in vivo* (5, 6) and *in vitro* (2-4). Priming and elongation are subsequently carried out by the host cell DNA polymerase α -DNA primase complex and a number of host factors including HeLa SSB¹ (7). T antigen, in addition to its origin binding capacity, has DNA helicase activity (8, 9). Owing to this activity, T antigen catalyzes an ATP-dependent unwinding of circular SV40 ori⁺ duplex DNA which can be extensive in the presence of an SSB and a topoisomerase. Previous studies have shown that the unwinding occurs bidirectionally from the origin (10), in keeping with the bidirectional replication of SV40 DNA (5, 6). It thus appears that following T antigen binding to the origin of replication, the protein can open the helix at the origin, allowing the DNA replication machinery access to the DNA, and subsequently can act as a helicase to unwind the helix in advance of the replication fork. This scheme is similar to the sequence of events that has been suggested in the initiation of DNA replication in *Escherichia coli* (11) and in bacteriophage λ (12). First, an origin-specific binding protein (*dnaA* gene product for *E. coli*, the O protein for λ) binds to the origin of replication, opening the helix and providing the *dnaB* gene product (DNA B protein) and other proteins access to the DNA. The DNA B protein helicase activity (13) can then unwind the DNA duplex in advance of the replication fork.

The helicase associated with SV40 T antigen can readily unwind duplex DNA regions that lack the SV40 ori⁺ sequence provided extensive single strands surround the duplex fragment (8, 9). Thus, T antigen can enter and unwind a duplex region that lacks an origin following binding to ssDNA. In addition to binding ssDNA, T antigen catalyzes the hydrolysis of ATP to ADP and P_i in a reaction that is stimulated by the presence of ssDNA (14-17). Such a triphosphatase activity that is stimulated or completely dependent on ssDNA is a general feature of DNA helicases (18). DNA helicases also usually show polarity as they traverse DNA strands. The polarity of helicases has been defined as the direction they move on the strand to which they bind. Thus, for example,

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¹ The abbreviations used are: SSB, ssDNA-binding protein; ssDNA, single-stranded DNA; bp, base pair; ATP γ S, adenosine 5'-O-(thiotriphosphate); NTP, nucleotide triphosphate.

the *uvrD* gene product, *E. coli* helicase II, translocates unidirectionally in a 3' to 5' direction (19) while the *E. coli* DNA B protein and the T7 gene 4 and the T4 gene 41 proteins move 5' to 3' (13, 20, 21).

In this report, we have extended the investigation of the T antigen-associated DNA helicase activity. This helicase translocates in the 3' to 5' direction and can act processively as it unwinds a duplex. The enzyme unwinds partial duplex DNA substrates containing as much as 0.8 kilobase pairs of duplex DNA. In addition, T antigen can unwind fully duplex fragments that lack single-stranded regions. Under appropriate conditions, this unwinding is dependent upon the presence of the SV40 origin of replication. A variety of nucleoside triphosphates support helicase activity on fully duplex fragments as well as on duplex regions flanked by extensive single strands.

MATERIALS AND METHODS

Enzyme Preparations—SV40 T antigen (4) and HeLa SSB (7) were prepared as previously described. *E. coli* SSB was either purchased from Pharmacia LKB Biotechnology Inc. or purified from *E. coli* bearing the SSB overproducer plasmid RLM55. Both the cell strain and the purification procedure were kindly provided by Dr. R. McMacken (Johns Hopkins University). Commercially acquired proteins and their sources included polynucleotide kinase (Boehringer Mannheim), bacterial alkaline phosphatase (Worthington), *E. coli* DNA polymerase I large fragment (Boehringer Mannheim or U. S. Biochemicals), and creatine phosphokinase (Sigma or Boehringer Mannheim). Restriction endonucleases were purchased from New England Biolabs. These enzymes were used as suggested by the supplier.

DNA Substrates—Circular M13mp7 ssDNA and replicative form I DNA were purified as described (19). DNAs from plasmids pSVO1ΔEP (4) (pBR322 derivative containing an intact SV40 origin of replication (*ori*⁺ DNA)), pSVLD6-1 (22) (containing a 6-bp deletion in the SV40 core origin), pOR1 (containing only T antigen-binding site 2 of the SV40 origin) (23), and pVOP and G39 (24) (containing the bacteriophage ϕ X174 origin of replication) (SV40 *ori*⁻) were also prepared as previously described (24). To prepare labeled duplex fragments, plasmid DNA (1–5 pmol) was restricted overnight and either 5'-end labeled with [γ -³²P]ATP upon treatment with bacterial alkaline phosphatase followed by polynucleotide kinase, or 3'-end labeled with [α -³²P]dNTPs by the large fragment of DNA polymerase I. All 3'-end labeling reactions were chased for 5 min with at least a 100-fold molar excess of each of the four unlabeled dNTPs to ensure that all recessed 3' termini were completely filled (25). The 311-bp fragment generated by *Eco*RI digestion of pSVO1ΔEP and the 291-bp fragments generated by *Bam*HI digestion of G39 or *Pvu*II digestion of pVOP were deproteinized with phenol/chloroform, ethanol precipitated, and either used directly in the fragment unwinding assay or were gel purified by electrophoresis through 3.5% polyacrylamide gels in TBE (0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA) prior to use. The 305-bp *Bst*NI fragment of plasmid pSVLD6-1 bearing a mutated SV40 origin and the 257-bp *Hha*I fragment of plasmid pOR1 bearing the wild type T antigen-binding site 2 sequence were gel purified from 3.5% polyacrylamide gels run in TBE. All DNA fragments isolated from polyacrylamide gels were eluted overnight in 0.5 M ammonium acetate, 1 mM EDTA and passed over an Elutip-D column (Schleicher and Schuell) following the manufacturer's protocol prior to use as substrate. The competitor DNA used in the duplex fragment unwinding assays consisted of plasmid pVOP DNA linearized with *Hinc*II.

Helicase Assay with Partially Duplex Substrates—The helicase assay measures the displacement of a [³²P]DNA fragment from circular M13mp7 ssDNA by T antigen. The DNA substrates used in helicase reactions were prepared as previously described (19) using either the 69-, the 341-, or the 849-bp *Hae*III restriction fragments isolated from a complete digest of M13mp7 replicative form I DNA. The DNA substrate used in the helicase assays to determine the direction of the unwinding reaction was prepared as described (19). Reaction mixtures (20 μ l) contained 40 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 5 mM dithiothreitol, 50 μ g/ml bovine serum albumin, 2.5% glycerol, 1.8 mM ATP, and approximately 0.2 μ M DNA (as nucleotides) as substrate. The reaction was incubated for 30 min (unless otherwise indicated) at 37 °C and terminated by adding 10 μ l of 33

mM EDTA, 25% glycerol, 3.3% sodium dodecyl sulfate, 0.5% xylene cyanol, 0.5% bromophenol blue. The reaction mixture was then loaded directly onto a 6% nondenaturing polyacrylamide gel as described (19). The polyacrylamide gel was analyzed by film autoradiography and quantitated by subsequent densitometric scanning of the x-ray film using a Kontes scanning densitometer. Quantitative data is expressed as the fraction of heat denaturable [³²P]DNA fragment displaced under the specified set of conditions.

Helicase Activity with Duplex DNA Fragments—The measurement of T antigen helicase activity with duplex DNA fragments was as follows: reaction mixtures (30 μ l) containing 25 fmol of ³²P *ori*⁺ or *ori*⁻ fragment DNA (350–400 cpm/fmol), 400 ng of unlabeled linear plasmid pVOP competitor DNA (where indicated), 40 mM creatine phosphate (diTris salt) (pH 7.7), 7 mM MgCl₂, 0.5 mM dithiothreitol, 25 μ g/ml creatine phosphokinase, 30 μ g/ml bovine serum albumin, 4 mM ATP, 0.6 μ g of *E. coli* SSB, and 0.8–1.2 μ g of SV40 T antigen were incubated for 2 h at 37 °C. Reactions were stopped by the addition of sodium dodecyl sulfate and EDTA to a final concentration of 0.1% and 20 mM, respectively. Mixtures were treated with proteinase K (10 μ g) for 1 h at 37 °C and electrophoresed through 5% nondenaturing polyacrylamide gels in TBE. Products were then visualized by autoradiography. Bands corresponding to the duplex fragment and the displaced strands were excised and counted in the presence of scintillation fluid. The percentage of radioactivity recovered in the displaced bands is indicated in most figures and legends. Backgrounds were usually 1–2% of the input substrates and have not been subtracted from the results presented.

RESULTS

The T Antigen Helicase Reaction—The helicase reaction catalyzed by T antigen was demonstrated using an assay that measured the ability of the enzyme to displace (unwind) short oligonucleotides annealed to M13 ssDNA (8, 9). We have further characterized this unwinding reaction using three different partial duplex DNA molecules constructed by annealing a specific restriction fragment of 71, 343, or 851 nucleotides in length to circular M13mp7 ssDNA. The specific restriction fragments used in the annealing reactions resulted from complete *Hae*III digestion of M13mp7 replicative form I DNA. Each restriction fragment annealed to a different

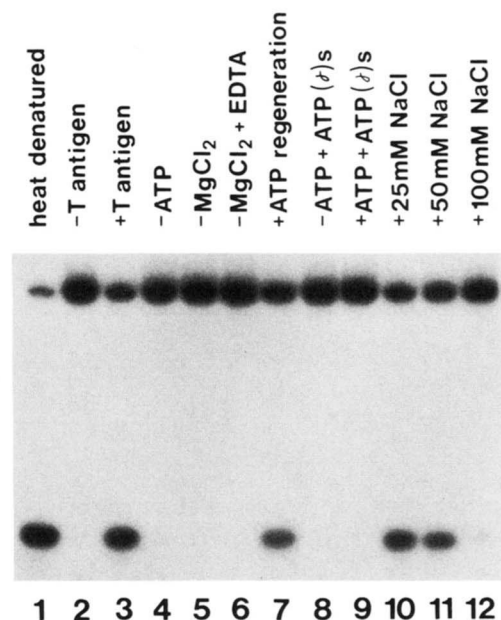


FIG. 1. Requirements for T antigen helicase activity with partial duplex substrates. Helicase activity was measured as described under "Materials and Methods" with the indicated additions or deletions using 150 ng of T antigen and the 71-bp partial duplex helicase substrate. When EDTA was used, it was present at a concentration of 5 mM. The ATP regeneration system consisted of creatine phosphate (50 mM) and creatine phosphokinase (100 μ g/ml). When ATP γ S was used, it was present at a concentration of 1.1 mM.

region on ssM13mp7 DNA and the final substrate molecule was circular with a single region of duplex DNA.

In the experiment presented in Fig. 1, a partial duplex DNA substrate containing 71 bp of duplex DNA was used to investigate the requirements of the T antigen helicase reaction. Using the standard reaction conditions, T antigen catalyzed the unwinding of approximately 75% of the annealed DNA fragment (Fig. 1, lane 3). The reaction required the presence of both ATP and $MgCl_2$ (Fig. 1, lanes 4 and 5). The addition of an ATP-regeneration system did not stimulate the unwinding reaction (Fig. 1, lane 7). In fact, the presence of creatine phosphate and creatine phosphokinase reproducibly inhibited the unwinding reaction to a small extent. This may be due to the fact that the unwinding reaction catalyzed by T antigen is relatively sensitive to ionic strength (Fig. 1, lanes 10–12). In the presence of 25, 50, and 100 mM NaCl, the displacement reaction was inhibited 7, 45, and 100%, respectively.

ATP γ S did not replace ATP in supporting the helicase reaction (Fig. 1, lane 8). Furthermore, the addition of ATP γ S to a complete reaction mixture inhibited the unwinding reaction almost quantitatively.

T Antigen Can Utilize Different NTPs as Substrates in the Helicase Reaction—The results presented in Fig. 1 indicate that ATP hydrolysis is required for helicase activity and provides the energy needed for movement along the DNA substrate and/or for disruption of the hydrogen bonds between the two strands of the DNA duplex. The other nucleoside triphosphates were also analyzed for their ability to support unwinding (Fig. 2). dTTP and dATP supported unwinding at about half the efficiency of ATP, while dCTP and UTP supported a 7-fold lower level of displacement relative to that detected with ATP. CTP, GTP, and dGTP failed to support any detectable unwinding.

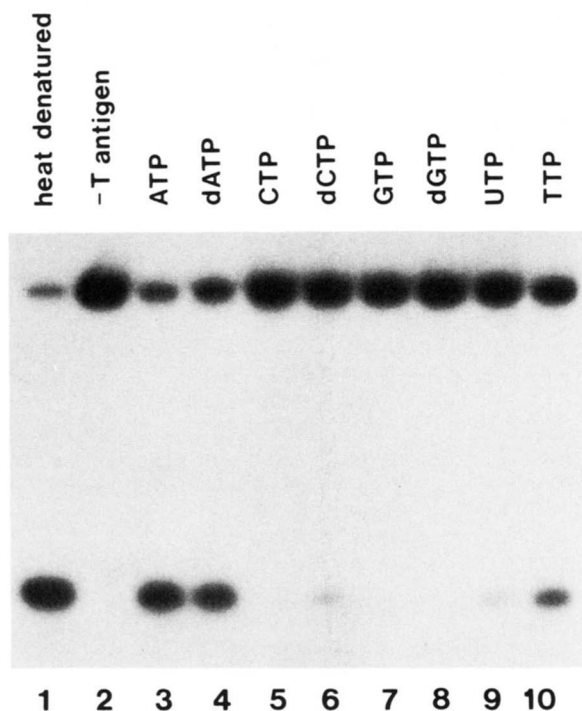


FIG. 2. T antigen utilizes several NTPs in the helicase reaction. Helicase activity was measured as described under "Materials and Methods" with the indicated NTP substituted for ATP using 150 ng of T antigen and the 71-bp partial duplex DNA substrate. Lane 1, heat denatured control; lane 2, no T antigen control; lane 3, 1.8 mM ATP; lane 4, 1.6 mM dATP; lane 5, 2 mM CTP; lane 6, 1.7 mM dCTP; lane 7, 2 mM GTP; lane 8, 2.1 mM dGTP; lane 9, 2 mM UTP; lane 10, 2 mM dTTP.

T Antigen Unwinds DNA in a 3' to 5' Direction—A number of helicases have been found to translocate in a 5' to 3' direction, while others translocate in a 3' to 5' direction, with the polarity defined as the direction the helicase moves on the strand to which it binds. To determine the direction of translocation of the T antigen, we have utilized a substrate designed specifically for this purpose (19) since the circular partial duplex constructs routinely used as helicase substrates do not allow for the determination of helicase directionality. This substrate contained a long, linear ssDNA molecule bearing short stretches of duplex DNA at both ends (Fig. 3A). Since T antigen, as do all helicases described to date, binds nonspecifically to ssDNA (26), the helicase should initially bind to the single-stranded region of this DNA molecule and then move through the duplex region. Unwinding in a 5' to 3' direction should result in the displacement of the 202-nucleotide DNA fragment, while unwinding in a 3' to 5' direction should cause displacement of the 143-nucleotide DNA fragment.

When T antigen was incubated with this substrate, the enzyme catalyzed the ATP-dependent unwinding of the 143-nucleotide DNA fragment (Fig. 3B, lanes 3–6), but not the 202-nucleotide DNA fragment. Since T antigen is able to move through and unwind at least 850 bp of duplex DNA (see Fig. 4), the 202-nucleotide DNA fragment does not represent too long a region of duplex DNA to be unwound. It thus appears that T antigen unwinds DNA unidirectionally in a 3' to 5' direction with respect to the single-stranded DNA on which it is bound.

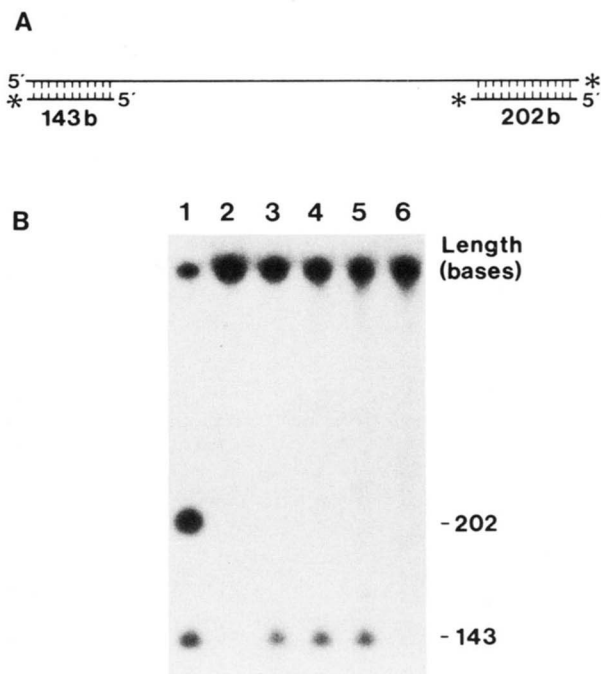


FIG. 3. T antigen unwinds duplex DNA in a 3' to 5' direction. Panel A, the linear partial duplex DNA substrate used to determine the direction of the T antigen unwinding reaction was constructed as described (19). The total length of the DNA molecule is 7200 nucleotides. Asterisks denote the position of radioactive label. Panel B, helicase activity was measured as described under "Materials and Methods" using the DNA substrate depicted in panel A except that the helicase reaction was terminated with 10 μ l of 45% glycerol, 45 mM EDTA, 0.1% bromophenol blue and xylene cyanol, 0.1% sodium dodecyl sulfate and proteinase K (60 μ g/ml). Incubation was continued for an additional 30 min at 37 $^{\circ}$ C. Lane 1, heat denatured control; lane 2, no T antigen control; lane 3, 75 ng of T antigen; lane 4, 150 ng of T antigen; lane 5, 300 ng of T antigen; lane 6, 150 ng of T antigen minus ATP.

The Length of Duplex DNA Unwound by T Antigen Is Independent of Protein Concentration—Previous reports (8, 9) showed that T antigen alone is capable of unwinding duplex regions up to 150 bp in length. We have investigated the effect of increasing lengths of duplex DNA on the helicase reaction catalyzed by T antigen using several partial duplex DNA substrates containing 71, 343, or 851 bp of duplex DNA annealed to M13mp7 ssDNA. Since the DNA concentration is almost the same for each substrate, the fraction of [32 P] DNA fragment displaced from one substrate can be compared to that displaced from another substrate (Fig. 4).

T antigen catalyzed the unwinding of a substantial fraction of the [32 P]DNA fragment from each of these DNA substrates in a reaction that showed a sigmoidal dependence on the protein concentration (Fig. 4). Low concentrations of T antigen catalyzed the unwinding of only a small fraction of the [32 P]DNA fragment. As the concentration of T antigen was increased the fraction of DNA fragment unwound increased and leveled off when approximately 70% of the fragment had been displaced. This sigmoidal response could be due to a cooperative interaction between T antigen molecules. Oligomerization of SV40 T antigen has been observed (27–30), although it is not clear at present whether this process is essential for the biological activities attributable to T antigen.

When the fraction of [32 P]DNA fragment displaced was compared for each partial duplex substrate at each T antigen concentration, it was found to be essentially the same (Fig. 4). Since there are approximately the same number of DNA substrate molecules in each reaction mixture and the assay only scores complete unwinding events, T antigen must have catalyzed the disruption of 12 times more hydrogen bonds in reactions utilizing the 851-bp partial duplex substrate than in reactions utilizing the 71-bp partial duplex substrate. This suggests that the basic enzymatic unit of T antigen moves through and unwinds whatever length of duplex DNA is encountered. The unwinding reaction does not require more protein to unwind longer duplex DNA and the length of duplex DNA unwound was not proportional with protein concentra-

tion. This is the type of processive reaction mechanism expected of a helicase involved in the replication of duplex DNA.

At present, it is not possible to determine exactly how many T antigen molecules might be contained in an enzymatic unit. At a T antigen concentration of 75 ng/20- μ l reaction mixture the ratio of molecules of T antigen to DNA substrate was approximately 10^3 :1. This is about 10-fold higher than the amount of enzyme added to reaction mixtures using prokaryotic helicases (19, 20). Since T antigen binds nonspecifically to ssDNA, a significant fraction of the T antigen present in the reaction mixture may be sequestered and not actively engaged in the helicase reaction. When this fact, together with the possible need for T antigen to oligomerize, is taken into account, the molar ratio of active T antigen enzymatic units to DNA substrate may be closer to the ratio observed using the prokaryotic helicases.

The Kinetics of the T Antigen Helicase Reaction—The kinetics of the helicase reaction catalyzed by T antigen were investigated using the three partial duplex DNA substrates described above (Fig. 5). The helicase reaction displayed a pronounced lag phase followed by a rapid increase in the rate at which the [32 P]DNA fragment was displaced from the circular ssDNA molecule. This lag phase may represent time required for T antigen monomers to assemble into oligomers on the DNA substrate or time required for T antigen to find the junction of single-stranded and duplex DNA. The lag phase was longer in reactions containing the 343-bp (or the 851 bp) than the 71-bp partial duplex substrate. After 10 min of incubation, slightly more than 35% of the 71-bp partial duplex substrate was unwound while only about 5% of the 343- or 851-bp partial duplex had been unwound. Thus complete unwinding of the 343- or 851-bp partial duplex molecule appeared to take longer than complete unwinding of the 71-bp partial duplex. Most of this difference, however, occurred during the lag period when no unwinding was detected. Once the unwinding reaction commenced, the rate of unwinding of all three substrates was almost identical. It is not clear why the lag periods observed with the 343- and 851-bp partial

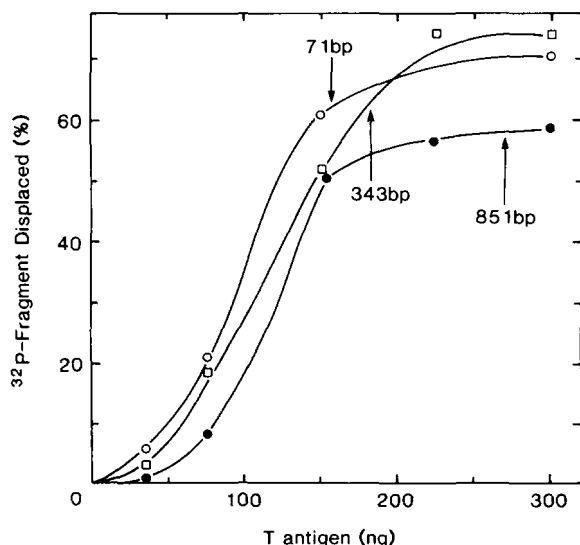


FIG. 4. Protein concentration dependence of the helicase reaction. Helicase activity was measured as described under "Materials and Methods" using the indicated partial duplex DNA substrates and the indicated concentration of T antigen. All incubations were for 30 min at 37 °C. In some cases the reaction mixture was increased to 160 μ l and 20- μ l aliquots were withdrawn at time intervals. Only the 30-min time point from these experiments have been included here. Each data point represents the average of four or more separate determinations.

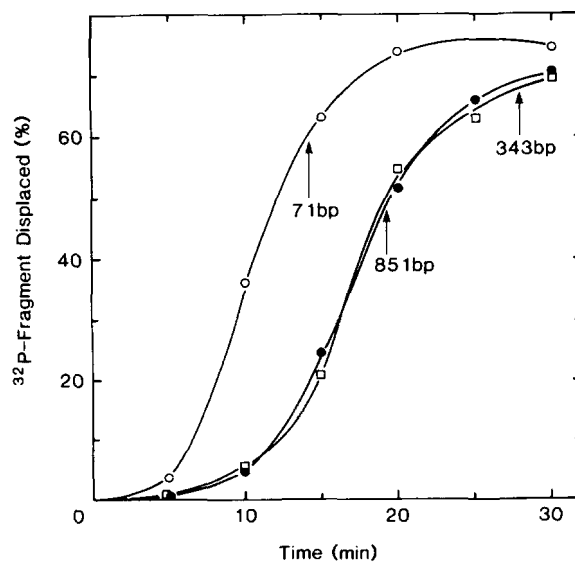


FIG. 5. Kinetics of the T antigen helicase reaction. Helicase activity was measured as described under "Materials and Methods" using the indicated partial duplex helicase substrate. The reaction mixture (160 μ l) contained 1.2 μ g of T antigen and aliquots (20 μ l) were withdrawn at the indicated times and loaded directly onto a nondenaturing polyacrylamide gel. Each point represents the average of three or more separate determinations.

duplex substrates were the same while the lag time noted with the 71-bp partial duplex substrate was only about half as long.

The extent of the unwinding reaction (achieved after 20 min with the 71-bp partial duplex and after 30 min on the other substrates) was essentially the same for each substrate. This is consistent with the results presented in Fig. 4 suggesting that the length of duplex DNA unwound was independent of protein concentration. If the length of duplex DNA unwound were strictly dependent on protein concentration then a lower reaction extent with increasing duplex length would be expected.

The Effect of *E. coli* SSB on the T Antigen Helicase Reaction—The single-stranded DNA-binding protein from *E. coli* (as well as other SSBs) has been shown to be essential for the unwinding of circular duplex DNAs containing the SV40 origin (10). For this reason it was of interest to see what effect this protein might have on the *in vitro* helicase reaction catalyzed by T antigen. Presumably SSBs are required to maintain DNA that has been unwound by a helicase in a single-stranded conformation. To test the effect of *E. coli* SSB on the T antigen helicase reaction, the binding protein was added prior to or after T antigen and the fraction of [³²P] DNA fragment displaced was determined at several different concentrations of SSB (Fig. 6). At the lowest concentration tested approximately 5% of the DNA substrate should be coated with SSB and at the highest concentration of SSB slightly more than 80% of the DNA should be coated with SSB. When *E. coli* SSB was added to the reaction mixture and allowed to bind ssDNA prior to the addition of T antigen, the helicase reaction was inhibited at all but the lowest concentration of SSB. These data suggest that either T antigen is unable to bind DNA in the presence of SSB or that T antigen is unable to displace bound molecules of SSB that it encounters when seeking regions of duplex DNA. The latter alternative seems more likely since concentrations of SSB that did not coat a substantial fraction of the ssDNA in the reaction mixture caused complete inhibition of the helicase reaction.

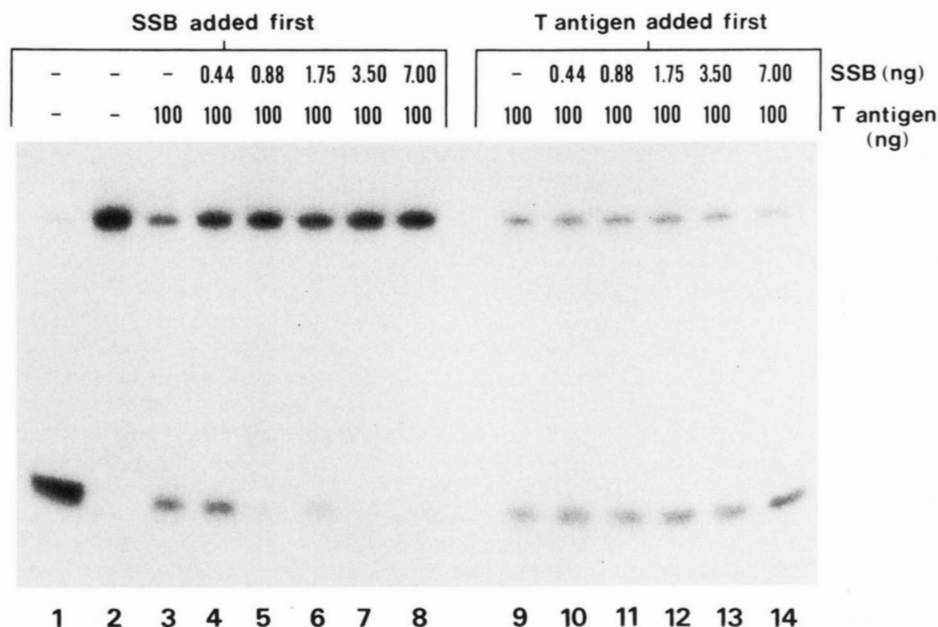
When T antigen was allowed to bind the DNA substrate prior to the addition of *E. coli* SSB, the helicase reaction was unaffected (Fig. 6). Only when the concentration of *E. coli* SSB was increased by another 5-fold was there an inhibition of the T antigen helicase reaction when T antigen was added to the DNA first (data not shown). In this latter case the

ssDNA should be completely coated with SSB and this may impede movement of bound T antigen molecules on the ssDNA molecule. Alternatively, the addition of excess SSB could force T antigen to dissociate from the DNA substrate. Taken together, these results suggest that once T antigen is bound to the DNA substrate the addition of *E. coli* SSB has little effect unless excess SSB is added.

Unwinding Activity with Duplex DNA Substrates—The substrates utilized in the preceding experiments all contained extensive single strands to which the T antigen could bind. It has also been shown that the T antigen is capable of unwinding fully duplex DNA that contains the SV40 origin of replication (9). This unwinding reaction is initiated at the origin of replication and proceeds bidirectionally on linear, relaxed circular and supercoiled circular DNA molecules (10). With circular ori⁺ DNA substrates, the T antigen ATP-dependent unwinding reaction requires an SSB and a topoisomerase that can remove positive superhelical turns (DNA gyrase or eukaryotic topoisomerases I or II). With linear substrates the unwinding reaction does not generate positive superhelical turns and therefore a topoisomerase is not required.

These observations suggested that duplex DNA fragments containing the SV40 origin sequence should be readily unwound. In contrast, duplex fragments lacking either an SV40 origin sequence with its T antigen-binding sites or ssDNA regions to which the T antigen could bind nonspecifically should not support helicase activity. To test this prediction two duplex fragments were prepared: a 311-bp fragment containing the SV40 origin, isolated by *Eco*RI digestion of pSV01ΔEP and a 291-bp fragment, isolated by *Bam*HI digestion of plasmid G39 (the *Hae*III 6b fragment of ϕX174 DNA cloned into the *Bam*HI site of pBR322). The same ϕX *Hae*III 6b fragment cloned into the *Pvu*II site of plasmid pVOP and obtained by *Pvu*II digestion was also used since it contained blunt ends. As shown in Fig. 7, duplex fragments with or without the SV40 origin sequence were displaced by T antigen with almost equality facility, although at low T antigen concentrations the reaction with the ori⁺ DNA fragment was more efficient. Up to 40–50% of the input fragments were converted to single-stranded structures. For reasons unclear, this yield was not increased by adding more T antigen or by longer incubation. The reaction with either DNA was dependent on ATP and T antigen, unaffected by ADP addition, and stimulated to the same extent by *E. coli* SSB (5- to 6-fold,

FIG. 6. The effect of *E. coli* SSB on the T antigen helicase reaction. Helicase activity was measured as described under "Materials and Methods" using the 343-bp partial duplex helicase substrate and the indicated amounts of T antigen and *E. coli* SSB. Reactions were terminated by the addition of 10 μl of 45% glycerol, 45 mM EDTA, 0.1% bromophenol blue and xylene cyanol, 0.1% sodium dodecyl sulfate and proteinase K (60 μg/ml). Incubations were continued for 30 min at 37 °C and then loaded directly onto a nondenaturing polyacrylamide gel. When SSB was added first, it was incubated with the DNA substrate in the complete reaction mixture minus T antigen for 3 min at 23 °C. When T antigen was added first, it was incubated with the DNA substrate in the complete reaction mixture for 3 min at 37 °C. The total time of incubation was 30 min. Lane 1, heat denatured control; lane 2, no T antigen control; lanes 3–14, *E. coli* SSB concentration was varied as indicated.



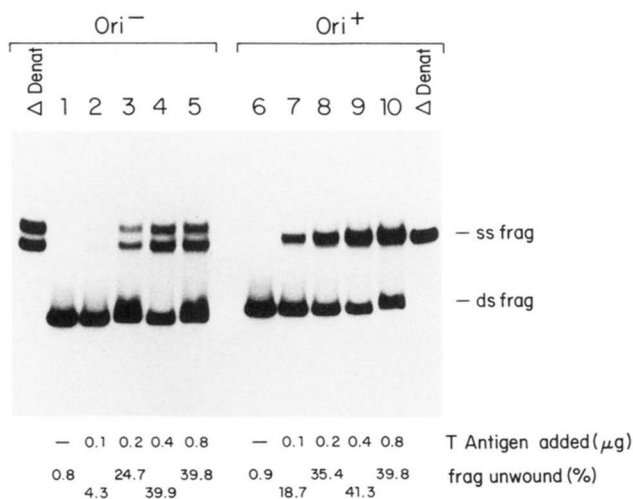


FIG. 7. The influence of T antigen concentration on the unwinding of ori⁺ and ori⁻ duplex DNA fragments. 3' ³²P-labeled ori⁺ fragments from plasmid pSV01ΔEP or ori⁻ fragments from plasmid G39 were incubated in the helicase assay with duplex fragments as described under "Materials and Methods." No competitor DNA was added and the T antigen concentration was varied as indicated. ds, double-stranded.

Table I). Furthermore, the ori⁻ fragment was displaced at almost the same rate as the ori⁺ fragment (Fig. 8). A 5' overhang was not required since both the *Pvu*II fragment of pVOP and the *Bam*HI fragment of G39 (both 5'-end labeled fragments and 3'-end labeled fragments filled in by the large DNA polymerase I fragment) were equally efficient substrates (not shown). The fact that both ori⁺ and ori⁻ DNA fragments were unwound differed from previous findings in which only ori⁺ circular or linear duplex DNAs were unwound (9, 10).

In contrast to the conditions used in the unwinding reaction with large duplex DNAs (9), displacement studies with DNA fragments were carried out with low amounts of DNA (0.2–0.6 ng) relative to the amount of T antigen (0.1–1 μg of the 82-kDa protein). Similar amounts of T antigen were used in both unwinding reactions, but reactions with full-length circular or linear ori⁺ or ori⁻ plasmid substrates contained 360 ng of DNA, nearly 1000-fold more than the amount used in the helicase assays described in Figs. 7 and 8. For this reason, helicase assays with the duplex fragments were repeated in reaction mixtures supplemented with unlabeled ori⁻ duplex DNA (competitor DNA). These conditions reduced the reaction with the ori⁺ DNA fragment only 30% while the activity with the ori⁻ DNA fragment was decreased markedly (Fig. 9). The greater decrease of activity observed with the ori⁻ fragments was evident even in the presence of excess T antigen (Fig. 10A). A variety of other duplex DNA plasmids lacking the SV40 origin (both linear and circular) were also tested as competitor DNA, and gave rise to similar inhibition of origin-independent unwinding.

The requirements for the displacement reaction were re-examined in the presence of competitor duplex DNA (Table I). In contrast to reactions observed in the absence of competitor DNA (the nonspecific unwinding activity), unwinding activity with ori⁺ fragments in the presence of competitor DNA was totally dependent on *E. coli* SSB. The HeLa SSB also supported unwinding, although higher levels were required for the reaction (Fig. 10B); the rate of unwinding using the competitor DNA was somewhat slower than the rate in the absence of the competitor (Fig. 8). The reaction was sensitive to salt (Table I); the unwinding reaction was significantly inhibited at 100 mM NaCl. The specific unwinding reaction was totally dependent on Mg²⁺ and in the presence

TABLE I

Requirements for unwinding of duplex fragments

Reactions containing either the ori⁺ or ori⁻ substrate were carried out as described under "Materials and Methods," except for the modifications noted. Requirements for the nonspecific reaction were analyzed under conditions of limiting T antigen (0.3 μg/reaction). Requirements for the origin-dependent reaction were analyzed using 0.8 μg of T antigen.

	Substrate unwound (%)	
	ori(+)	ori(-)
Nonspecific reaction (no competitor DNA)		
Complete	25.3	20.1
–T antigen	0.9	0.6
– <i>E. coli</i> SSB	4.6	3.9
–Mg ²⁺	1.1	1.1
–ATP	1.3	0.9
+2.5 mM ADP	22.1	17.3
– <i>E. coli</i> SSB + HeLa SSB (1 μg)	23.9	16.3
Origin-dependent reaction (with competitor DNA)		
Complete	42.1	
–T antigen	0.4	
– <i>E. coli</i> SSB	1.6	
–MgCl ₂	1.3	
–ATP	1.4	
+20 mM NaCl	42.0	
+50 mM NaCl	33.5	
+100 mM NaCl	10.1	
–MgCl ₂		
+0.8 mM MnCl ₂	20.1	
+4 mM MgSO ₄	38.1	
+4 mM Mg(C ₂ H ₃ O ₂) ₂	40.0	

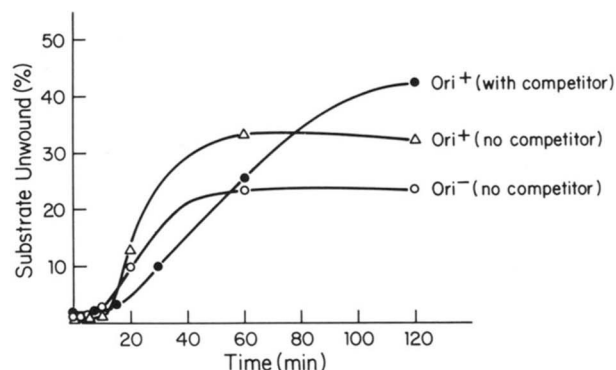


FIG. 8. Influence of time on the unwinding of duplex DNA fragments. Reactions, with or without 400 ng of linearized plasmid pVOP competitor DNA, were as described under "Materials and Methods." Reactions lacking competitor DNA were carried out utilizing 0.3 μg of T antigen, while reactions containing competitor were analyzed using 0.8 μg of T antigen.

of 4 mM ATP, the reaction was saturated at 4 mM MgCl₂. Mn²⁺ also supported unwinding (Table I), with an optimal efficiency of 50% of that seen with Mg²⁺ at a MnCl₂ concentration of 0.8 mM (not shown).

Mutant Origin Fragments As Substrates for the Unwinding Reaction—Deb et al. (31) have shown that a 64-bp core origin of replication, consisting of T antigen-binding site 2 and flanking sequences, is sufficient for T antigen-dependent DNA synthesis *in vivo*, and a similar observation was made *in vitro*.² Deletion of one of the 4 pentanucleotide T antigen

² F. B. Dean, J. A. Borowiec, Y. Ishimi, S. Deb, P. Tegtmeyer, and J. Hurwitz, unpublished results.

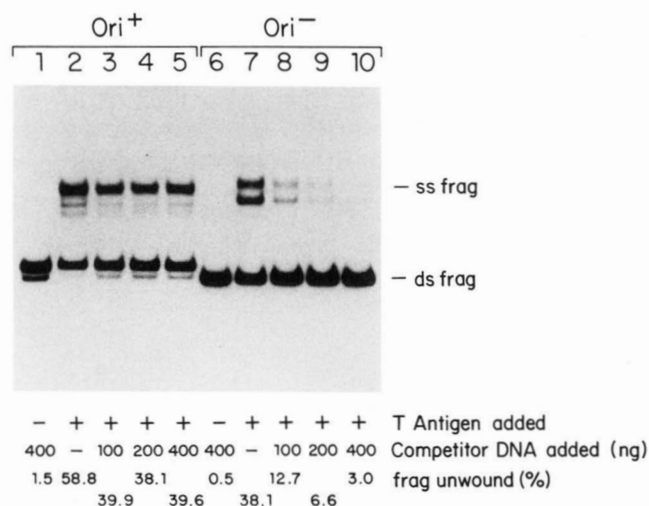


FIG. 9. Influence of competitor DNA concentration on the unwinding of duplex ori⁺ and ori⁻ DNA fragments. Reactions containing 1.2 μ g of T antigen were carried out as described under "Materials and Methods" except that the concentration of competitor pVOP replicative form III DNA added was varied as indicated.

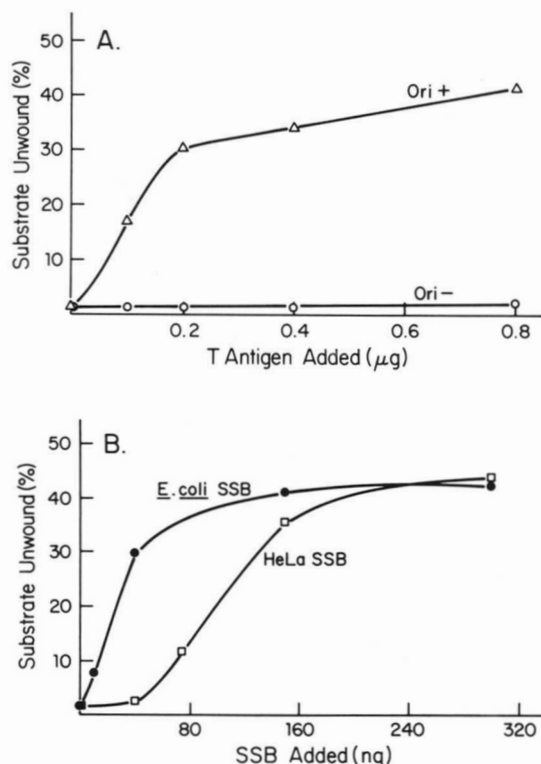


FIG. 10. Effect of increasing protein concentration on the unwinding reaction. A, effect of SV40 T antigen concentration on the unwinding reaction carried out in the presence of competitor DNA. Reactions, containing 400 ng of competitor DNA and 0.6 μ g of *E. coli* SSB, were carried out as described except that the T antigen concentration was varied as indicated. B, influence of *E. coli* and HeLa SSBs on the unwinding reaction of the ori⁺ DNA fragment in the presence of competitor DNA. Reactions containing the ori⁺ DNA fragment substrate, 400 ng of carrier DNA, and 1.0 μ g of T antigen were carried out as described under "Materials and Methods," except that SSB was added as indicated.

contact sites from binding site 2 of a complete SV40 origin rendered the mutant origin defective for replication both *in vivo* (22) and *in vitro* (9). Similar results were obtained with the plasmid unwinding assay; the core origin allowed efficient unwinding while the origin bearing the deletion supported only minimal unwinding (10). Since the fragment unwinding

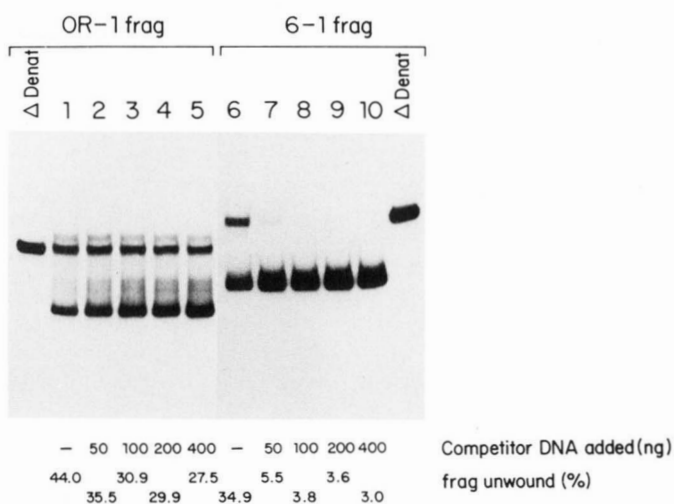


FIG. 11. Unwinding of SV40 origin mutant DNA fragments by SV40 T antigen. Reactions were carried out as described in the legend to Fig. 9 with the exception that the 3' ³²P-labeled OR-1 and 3' ³²P-labeled SVLD6-1 fragments were used in place of the ori⁺ wild type fragment. The source and structure of these two DNA fragments is described in the text.

TABLE II

Effect of various nucleotides on the unwinding of duplex fragments (origin-dependent reaction)

Reactions contained 25 fmol of 3' ³²P end-labeled *Eco*RI digest of plasmid pSV01ΔEP (ori⁺) as substrate, 400 ng of competitor ori⁻ DNA, and 0.8 μ g of SV40 T antigen. Except where noted, 4 mM of the indicated nucleoside triphosphate(s) was added. All other conditions were as described under "Materials and Methods."

Nucleoside triphosphate added	Substrate unwound %
None	2.5
ATP	41.8
ATP, omit T antigen	1.4
GTP	2.6
UTP	19.4
CTP	3.7
dATP	26.0
dGTP	3.6
dTTP	40.5
dCTP	20.1
CTP + ATP (0.5 mM)	25.6
ATP (0.5 mM)	27.1

assay in the presence of competitor DNA was origin dependent, we wanted to know how fragments bearing the core origin (isolated from plasmid pOR1) or the origin with the deleted pentanucleotide (isolated from plasmid pSVLD6-1 (22)) would behave in this assay. As shown in Fig. 11, both fragments supported unwinding when no competitor DNA was present. Addition of competitor DNA did not affect unwinding of the pOR1 fragment, but as expected, inhibited unwinding of the pSVLD6-1 derived fragment. Thus, origins incapable of supporting replication also fail to support origin-dependent unwinding as measured by either the plasmid unwinding or the specific fragment unwinding assay.

Influence of Nucleoside Triphosphate on the T Antigen Unwinding Assay with Duplex Fragments—The helicase activity of SV40 T antigen requires high concentrations of ATP. Even in the presence of an ATP-regenerating system, 4 mM ATP was required to saturate unwinding reactions containing 4 mM MgCl₂. A similar ATP saturation curve was obtained when the molar ratio of ATP to MgCl₂ was maintained at 1:1 over the range examined (not shown).

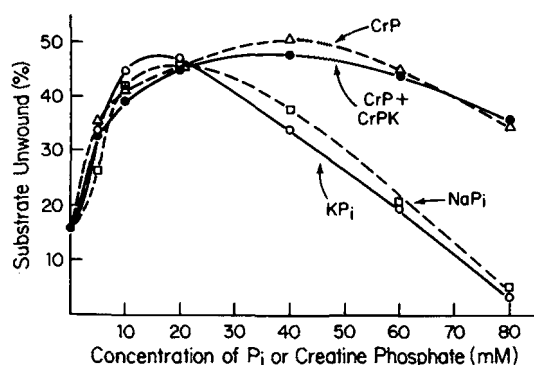


FIG. 12. Influence of increasing concentrations of phosphate or creatine phosphate on the displacement of ori^+ duplex DNA fragments. Reaction mixtures lacking phosphate or creatine phosphate buffer contained 40 mM Tris-HCl buffer (pH 7.6) and 1.1 μg of T antigen. As the concentration of the indicated phosphate buffer was increased, the amount of Tris buffer added was reduced by a comparable amount. Thus, when 5 or 20 mM phosphate (or creatine phosphate) was added, 35 or 20 mM Tris buffer was present. No additional Tris buffer was added at phosphate (or creatine phosphate, CrP) concentrations above 40 mM. Creatine phosphokinase (CrPK) (25 $\mu\text{g}/\text{ml}$) was added where indicated. All other conditions were as described under "Materials and Methods." Phosphate buffers were adjusted to pH 7.6.

The other nucleoside triphosphates were tested for their ability to support the unwinding of the ori^+ DNA fragments (Table II). Maximal activity was obtained with dTTP as well as ATP while dATP, dCTP, and UTP were 50–60% as effective as ATP and dTTP. CTP, GTP, and dGTP were only marginally effective. The low activity observed with CTP was not due to the presence of an inhibitor since reactions carried out with CTP plus limiting concentrations of ATP readily supported as much unwinding as reactions containing only the limiting concentration of ATP. The ability of the various nucleoside triphosphates to support origin-dependent fragment unwinding was qualitatively similar to their ability to support unwinding on the partial duplex M13mp7 substrates (Fig. 2), consistent with the assumption that the two assays are measuring the same activity.

Influence of Phosphate on the Unwinding Reaction—The replication of circular ori^+ duplex DNA by crude extracts is dependent on the presence of creatine phosphate and creatine phosphokinase (2, 7). The reaction leading to the unwinding of the ori^+ circular duplex DNA also exhibited a dependence on creatine phosphate (9). It was assumed that the unwinding reaction was similarly dependent on the regeneration of ATP. This proved to be incorrect. To our surprise, the presence of the creatine phosphate/creatine phosphokinase-regenerating system was found to stimulate unwinding with dTTP despite the fact that creatine phosphokinase is specific for ATP (32). Subsequently, we determined that the unwinding reaction requires a substantial concentration of phosphate ions and creatine phosphate can work in lieu of phosphate. As shown in Fig. 12, unwinding of ori^+ fragments increased as the creatine phosphate concentration was elevated and this occurred in the presence or absence of creatine phosphokinase. Similar increments occurred when phosphate buffer was used in place of creatine phosphate. The major difference noted was that high concentrations of phosphate were inhibitory while high concentrations of creatine phosphate were not. The activation of the displacement reaction with creatine phosphate was not dependent on the presence of creatine phosphokinase. The T antigen preparations used were screened for this kinase, but none was detected.³ In view of

the effect of phosphate ion on the reaction, we analyzed the effect of other anions on the fragment unwinding reaction. Neither sulfate nor acetate ions appeared to influence the reaction, as substitution of MgSO_4 or $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ for MgCl_2 did not affect the extent of unwinding (Table I).

DISCUSSION

Purified T antigen exhibits several different activities *in vitro* including sequence-specific binding to double-stranded DNA (15), nonspecific binding to ssDNA (26), ATPase activity (14), and helicase activity (8, 9). Consistent with these different activities, T antigen has been shown to have several different roles in the life cycle of the SV40 virus, including a role(s) in DNA replication (33). Recent evidence suggests that T antigen acts at the SV40 origin of replication by binding to specific DNA sequences and then, in the presence of a topoisomerase and SSB, unwinds the DNA duplex from the replication origin (9, 10).

The results presented here show that T antigen can act as a general helicase that unwinds DNA in a 3' to 5' direction. This has been demonstrated using a linear ssDNA substrate with duplex ends. This is the same direction of translocation exhibited by *E. coli* helicase II (19), *E. coli* rep protein (34), and the *E. coli* 75-kDa helicase (35). None of these helicases are thought to be the primary helicase involved in DNA replication in *E. coli*. It is interesting to note that the DNA helicases known to be directly involved in DNA replication, the T7 gene 4 protein, the T4 gene 41 protein, and the *E. coli* DNA B protein, all unwind DNA in the 5' to 3' direction (13, 20, 36). Thus T antigen appears to be the first helicase known to be involved in DNA replication that migrates in a 3' to 5' direction. If it is assumed that T antigen also moves 3' to 5' on the ori^+ duplex DNA, the DNA polymerase α -DNA primase complex could synthesize an RNA primer that could be elongated directly behind the T antigen. Thus any agent that blocked the movement of the T antigen would block elongation of DNA chains. Stahl *et al.* (33) have shown that antibodies that inhibit the T antigen NTPase activity block the *in vitro* elongation of DNA chains initiated on SV40 DNA *in vivo*. This suggests that T antigen plays an important role both in the initiation and elongation reactions. Wiekowski *et al.* (37) have further shown that monoclonal antibodies that inhibit helicase activity also strongly inhibit both T antigen ATPase activity and *in vitro* DNA replication. Thus the ATPase activity being measured is clearly involved in the unwinding process.

The rate of nucleotide incorporation in the *in vitro* replication reaction begins with a lag, as do all unwinding reactions in which T antigen participates. And since the lag in DNA synthesis can be abolished by preincubation of ori^+ DNA with T antigen, ATP, HeLa SSB, and the creatine phosphate/creatine phosphokinase-regenerating system,⁴ it is likely that the lag in replication is due to the same phenomenon responsible for the lag detected in the various unwinding reactions. The reason(s) for the lag in the unwinding of the various substrates tested may be similar. Recent electron microscopic studies have suggested that following successive T antigen monomer binding to recognition sequences at the origin, multimeric T antigen structures assemble (38). A number of other laboratories have also shown that T antigen can oligomerize (27–30), although it is not yet clear what constitutes the active T antigen species. If a multimeric form of T antigen is in fact required for helicase activity, the oligomerization process may take time to occur and may account for the lag in unwinding seen with the various substrates. The opening

³ J. Hurwitz, unpublished results.

⁴ C. R. Wobbe and J. Hurwitz, unpublished results.

of the helix may also contribute to the lag phase. Although T antigen binding to the replication origin appears to be rapid (15), the subsequent invasion of the helix may be considerably slower. Likewise, the invasion of a duplex region by ssDNA-bound T antigen may be less rapid than the initial binding event. Once in the duplex region, T antigen appears to act processively as a helicase. The rates of displacement of a 71-nucleotide duplex and an 850-nucleotide duplex region were almost identical once the lag period ended.

The characteristics of the T antigen displacement reaction varied depending upon the concentration and structure of the substrate used. All duplex fragments were unwound when only 0.6 ng of DNA was present in the reaction. Since T antigen binds to single-stranded regions nonspecifically, it is possible that at such a high ratio of T antigen to DNA, melting at the ends of the DNA fragments can generate single-stranded regions capable of binding T antigen. The unwinding of the ori⁻ DNA could not be ascribed to the creation of single-stranded regions by means of nucleolytic degradation since the size of the ssDNA products of the unwinding reaction were identical to the input substrate (after denaturation) when the two were run side by side on a sequencing gel (data not shown). When the ratio of T antigen to DNA was reduced approximately 1000-fold by the addition of competitor DNA, only ori⁺ fragments were unwound and this unwinding required SSB and was stimulated by phosphate or creatine phosphate. In the absence of SSB, there was no detectable unwinding reaction. These requirements may explain the discrepancy between the results presented here and those of Stahl *et al.* (8). They reported that a duplex fragment containing the SV40 origin was not unwound by T antigen. In their experiments, SSB and phosphate were not added.

The unwinding of both the ori⁻ duplex substrates and the partial duplex substrates showed a sigmoidal response to T antigen. Little unwinding occurred at low T antigen concentrations (Figs. 4 and 7). This sigmoidicity may be due to the need for a critical T antigen concentration before oligomerization of non-origin bound T antigen can occur. A high concentration of T antigen may also be required for the invasion of fragment ends. Furthermore, the long single-stranded regions of the partial duplex substrates probably result in the sequestration of large amounts of T antigen. In contrast to the sigmoidicity observed with the ori⁻ substrates, the displacement reaction with ori⁺ DNA fragments (with or without competitor) showed little sigmoidicity at the concentrations tested (Figs. 7 and 10A). This is consistent with the high affinity exhibited by T antigen for the multiple binding sites at the origin region (31) and suggests that the origin sequence can be efficiently utilized by T antigen as an entry site to the duplex DNA.

In an attempt to overcome the sequestration of T antigen by single-stranded regions, reactions with partial duplex substrates were carried out in which *E. coli* SSB was first bound to the DNA and then T antigen was added. This procedure resulted in no detectable helicase activity. In contrast, when T antigen was first incubated with the DNA substrate, and then *E. coli* SSB added, T antigen-dependent helicase activity was readily detected. This suggests that T antigen cannot translocate through ssDNA-*E. coli* SSB complexes. In the unwinding reaction starting at the SV40 duplex origin, SSB must act on the single strands generated after helicase action, thus avoiding translocation of T antigen through SSB-ssDNA complexes.

The origin-dependent and single strand-dependent helicase reactions show almost identical specificity regarding the nucleoside triphosphate that supported helicase action. ATP and dTTP were the most effective nucleotides while dATP,

dCTP, and UTP supported a reduced level of unwinding. The nucleotides CTP, GTP, and dGTP were virtually inactive. It is interesting to note that other helicases have been shown to use a variety of nucleotides other than ATP. dTTP, for example, is efficiently utilized by the T7 gene 4 helicase (20), while the common ribonucleoside triphosphates support the helicase activity of the DNA B protein (13).

The nucleoside triphosphatase activity associated with SV40 T antigen was investigated in light of the nucleotide specificity of the helicase reaction. T antigen preparations hydrolyzed all nucleoside triphosphates including CTP, dGTP, and GTP. Their hydrolysis to the corresponding di-phosphates were stimulated by poly(dT).³ The NTPase activity of helicases, however, is known to be influenced by the DNA effector present in the reaction. The *E. coli* rep protein, for example, can hydrolyze GTP and dGTP at about one-third the efficiency of ATP when ssDNA is used as effector, but is unable to hydrolyze these purine substrates at a replication fork (39). An unwinding fork may similarly restrict the NTPase activity of T antigen. The coupling between NTP hydrolysis and helicase activity remains to be further elucidated.

It is clear that the T antigen helicase activity plays an important role in the replication of SV40 DNA. The multiple activities intrinsic to T antigen should make this bidirectional replication pathway less complicated than other systems in which separate origin binding proteins and a separate DNA helicase protein are involved. It is interesting to note that in the T7 phage system, both primase and helicase are intrinsic activities associated with the T7 gene 4 protein (20). There is also an association between the primase (gene 61) and helicase (gene 41) coded by the T4 phage system (19, 36). The relationship between the primase and the helicase in these phage systems might lead to the prediction of a similar relationship between SV40 T antigen and DNA primase. Recently, an interaction between T antigen and DNA polymerase α has been detected by immunologic procedures (40, 41). Since DNA polymerase α and DNA primase usually occur as a complex (42-45), it is possible that these findings reflect an interaction between primase and T antigen rather than DNA polymerase α and T antigen. Further characterization of the interaction between T antigen and these enzymes should answer this question.

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